Developmental Biology **212,** 455–464 (1999)
Article ID dbio.1999.9374, available online at http://www.idealibrary.com on IDEAL®

Xenopus by Homeodomain Factors Dlx3 and Msx1

Jules A. Feledy, Maureen J. Beanan, John J. Sandoval, Jennifer S. Goodrich,[1] Jae H. Lim,* Mami Matsuo-Takasaki,* Sheryl M. Sato,* and Thomas D. Sargent[2]

*Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, and *Genetics and Biochemistry Branch, National Institute of Diabetes, Digestive and Kidney Disease, National Institutes of Health, Bethesda, Maryland 20892*

Patterning of the embryonic ectoderm is dependent upon the action of negative (antineural) and positive (neurogenic) transcriptional regulators. Msx1 and Dlx3 are two antineural genes for which the anterior epidermal–neural boundaries of expression differ, probably due to differential sensitivity to BMP signaling in the ectoderm. In the extreme anterior neural plate, Dlx3 is strongly expressed while Msx1 is silent. While both of these factors prevent the activation of genes specific to the nascent central nervous system, Msx1 inhibits anterior markers, including Otx2 and cement gland-specific genes. Dlx3 has little, if any, effect on these anterior neural plate genes, instead providing a permissive environment for their expression while repressing more panneural markers, including prepattern genes belonging to the Zic family and BF-1. These properties define a molecular mechanism for translating the organizer-dependent morphogenic gradient of BMP activity into spatially restricted gene expression in the prospective anterior neural plate.

Key Words: **Msx1; Dlx3; *Xenopus;* antineural patterning.**

INTRODUCTION

In *Xenopus*, the embryonic ectoderm is initially specified as epidermis (Sudarwati and Nieuwkoop, 1971). This identity is dependent upon cell–cell signaling via bone morphogenetic proteins (BMPs; Hogan, 1996; Graff, 1997). During gastrulation, dorsalizing factors secreted from the Spemann organizer, including noggin (Zimmerman *et al.*, 1996), chordin (Piccolo *et al.*, 1996), and follistatin (Fainsod *et al.*, 1997), antagonize BMP signaling by binding to extracellular BMP and preventing interactions with cognate BMP receptors. Disruption of the BMP feedback loop initiates the process of neural induction, resulting in the suppression of epidermal-specific genes, such as epidermal keratin, and the activation of regulatory and structural genes specific to neural tissues (Hemmati-Brivanlou and Melton, 1997). During neural induction, the ectoderm is thus partitioned into its primary derivatives, presumptive epidermis on the ventral side and presumptive central nervous system (CNS) on the dorsal side.

The switch from epidermis to CNS is mediated by a cascade of regulators, beginning with prepattern factors, including the Zic family of zinc finger proteins (Nakata *et al.*, 1997; Brewster *et al.*, 1998; Mizuseki *et al.*, 1998) which appear to promote specification of the neural plate and neural crest. Prepattern factors in turn activate proneural genes, including those encoding basic helix-loop-helix transcription factors such as neurogenin (Ma *et al.*, 1996), neuroD (Lee *et al.*, 1995), and vertebrate homologs of the *Drosophila* achaete-scute gene (XASH-3; Zimmerman *et al.*, 1993). The proneural genes function to organize the neural plate into regions that will give rise to specific types of neurons.

In addition to these positively acting factors, another class of genes that is important in specifying ectodermal fate comprises a relatively small number of antineural or ventralizing genes, including the homeobox genes Msx1 and PV.1/Xvent and the zinc-finger gene Gata1b (Suzuki *et al.*, 1997; Ault *et al.*, 1997; Onichtchouk *et al.*, 1998; Xu *et al.*, 1997). Overexpression of these genes promotes a ventral phenotype in the ectoderm and inhibits neuralization, suggesting a fundamental role in the negative regulation of

[1] Present address: Department of Molecular Biology, Princeton University, Princeton, NJ 08544.

[2] To whom correspondence should be addressed at Bldg. 6B, Room 412, NIH MSC 2790, 6 Center Drive, Bethesda, MD 20892-2790. E-mail: tsargent@nih.gov.

neural differentiation (Sasai, 1998). In this report we focus on Msx1 and another ventrally expressed homeodomain gene, Dlx3.

Msx1, a vertebrate homolog of the *Drosophila msh* homeobox gene (Su *et al.*, 1991), is one of three genes which comprise the Msx family of transcriptional repressors. This gene has been shown to be an immediate early response to BMP4 signaling in *Xenopus* ectodermal cells and to block neuralization elicited by overexpression of dominant-negative BMP4 receptor (tBR) in ectodermal explants (Suzuki *et al.*, 1997). Dlx3, a member of the Distal-less family of homeodomain genes, which was originally identified in *Drosophila* (Cohen *et al.*, 1989), has an expression pattern that suggested it might play an antineural role similar to that of Msx1 (Dirksen *et al.*, 1994). Like Msx1, Dlx3 is activated at the beginning of gastrulation, and its expression is excluded from the dorsal region of the embryo, the future neural plate. By the neurula stage, ectodermal expression of Dlx3 is essentially epidermis-specific. This gene has also been shown to play a role in the differentiation of epidermal cells in the mouse (Morasso *et al.*, 1996). Based on these observations we hypothesized that Dlx3 could act in the early *Xenopus* embryo to promote epidermis and to repress neural development.

In this report we show that Dlx3, like Msx1, functions as an antineural regulator. Msx1 and Dlx3 exhibit different expression patterns in the extreme anterior neural plate during gastrulation, and we present evidence supporting the conclusion that this is due to differential sensitivity to attenuation of BMP morphogenic signals. We show that the antineurogenic effects of Msx1 are preferential for anterior targets and that this inhibition is complemented by the antineurogenic properties of Dlx3, which preferentially blocks the activation of Zic-class prepattern genes. We propose a model through which Dlx3 and Msx1 function to mediate the response of anterior neural plate cells to morphogenic signaling from the organizer.

MATERIALS AND METHODS

Preparation of embryos and ectodermal explants. *Xenopus laevis* eggs were collected, fertilized *in vitro*, dejellied with 2% cysteine, pH 7.8, and transferred to dechlorinated water until injection. Embryos were staged according to Nieuwkoop and Faber (1967). Following injection, the embryos were cultured in 3% Ficoll/1× MMR for 60–90 min and then transferred to 0.3× MMR (1× MMR is 0.1 M NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 0.1 mM EDTA, 10 mM Hepes, pH 7.4). For animal explants, approximately 2/3 of the pigmented ectoderm was dissected from stage 7–8 embryos. Care was taken to avoid marginal zone tissue or loosely adherent mesoderm cells. Caps were dissected in 0.3× MMR and cultured in the same buffer with 50 μg/ml gentamycin (Gibco BRL, Gaithersburg, MD). For the cycloheximide experiment, animal caps were dissected at stage 7–8, cultured in 5 μg/ml cycloheximide or in 0.3× MMR until stage 11, and then processed for RNA. In all experiments, a minimum of 30 explants were pooled for each RNA sample.

Constructs. The pSP64T-△ tBR encodes a murine type I BMP4 receptor truncated in the cytoplasmic domain (Suzuki *et al.*, 1994). The $pCS2^+$-Zic-r1 construct was a gift from Dr. Y. Sasai. The Dlx3 and Msx1 plasmids were subcloned in $pCS2^+$ (Turner and Weintraub, 1994) modified to contain a T7/SP6 dual promoter region and an optimal translation initiation site (Kozak, 1987). The $pCS2^+$ T7/SP6-Dlx3 contained the Dlx3 open reading frame fragment (831 bp) which was generated by PCR from template with the primers GAG GAG GAA TTC ATG AGT GGC CCC TAT GAG AAT and GAG GAG GGA TCC GCG ATA CAC TGT ATC GGG AGG AGG and subcloned into $pCS2^+$ T7/SP6. The $pCS2^+$ T7/SP6-Msx1 contained a Msx1 fragment (819 bp) initiating at the second methionine in the open reading frame, which most closely matches the presumptive amino-terminal sequence of other vertebrate Msx1 polypeptides (Davidson, 1995). This fragment was generated by PCR with primers GAG GAG ATC GAT GCC ATG GCT TCT TAC CAG CCT GGG and CTC CTC TCT AGA CTA GGA CAG ATG GTA CAT GCT GTA and subcloned into $pCS2^+$ T7/SP6. Nucleotide fidelity was verified by sequencing using Sequenase (US Biochemical, Inc., Cleveland, OH). The Dlx3 and Msx1 constructs were translated *in vitro* with approximately equal efficiency, as determined by [^{35}S]methionine incorporation and SDS–polyacrylamide gel electrophoresis (data not shown).

In vitro transcription. Capped sense RNAs for microinjection were transcribed using mMessage Machine kits according to the manufacturer's instructions (Ambion, Austin, TX). Dlx3 and Msx1 plasmids were linearized with *Asp*718 and transcribed with T7 polymerase. SP64T-△tBR was linearized with *Eco*RI and transcribed with SP6. $CS2^+$-Zic3 was linearized with *Asp*718 and transcribed with SP6 polymerase. RNA concentrations were determined spectrophotometrically and RNA size and concentrations were verified by methylmercury hydroxide agarose gel electrophoresis (Bailey and Davidson, 1976).

In situ hybridizations and sectioning. *In situ* hybridization studies were performed on albino embryos as described previously with minor modifications (Harland, 1991). Antisense digoxigenin-labeled and fluorescein-labeled probes were synthesized using the Boehringer Mannheim (Indianapolis, IN) RNA labeling kit following the manufacturer's instructions. Embryos were incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim), stained with BM purple (purple color; Boehringer Mannheim), and photographed. For double *in situ* hybridizations, second chromogenic reactions were performed after denaturation of primary antibodies, incubated with alkaline phosphatase-conjugated anti-fluorescein antibody (Boehringer Mannheim), stained with BCIP alone (turquoise blue color; Promega, Inc., Madison WI), and photographed. For cryosectioning, fixed embryos were infiltrated with 30% sucrose in 1× PBS and embedded in OCT compound (Miles, Inc. Elkhart, IN). Coronal sections (20 μm) were mounted on slides coverslipped with Aqua Poly/Mount (Polysciences, Inc., Warrington, PA) and photographed.

Microinjection and RNA analysis. For targeted overexpression, 300 pg of β-gal mRNA alone or mixed with 40–60 pg of Dlx3 mRNA was co-injected into one of the two dorsal blastomeres at the 8- to 16-cell stage. Embryos were fixed at stage 17 with 4% paraformaldehyde and stained for β-galactosidase activity using salmon-gal (6-chloro-3-indoxyl-β-D-galactopyranoside; Biosynth International, Naperville, IL). For animal cap experiments a total of 2 ng of RNA encoding tBR was injected alone or in combination with Dlx3 (120 pg), Msx1 (120 pg), or Zic3 (100 pg) RNA into two sites in the animal hemisphere of the 1-cell embryo. Animal caps were removed at stage 7–8 and cultured until the appropriate stage. RNA isolation, methylmercury hydroxide gel electrophoresis, and

Northern blot analysis for expression of Dlx3, Msx1 (Su *et al.,* 1991), CG7 (Jamrich and Sato, 1989), Otx2 (Pannese *et al.,* 1995; Blitz and Cho, 1995), Xag1 (Sive *et al.,* 1989), BF-1 (Bourguignon *et al.,* 1998), Zic3 (Nakata *et al.,* 1997), Nrp1 (Richter *et al.,* 1990), XK81 (Jonas *et al.,* 1985), Xbra (Smith *et al.,* 1991), and 18S were as previously described (Sargent *et al.,* 1986).

RESULTS

Expression Patterns of Dlx3 and Msx1

Whole-mount *in situ* hybridization was used to examine the embryonic distribution of Dlx3 and Msx1 gene expression. The observed expression patterns for both genes were similar to previously published results (Dirksen *et al.,* 1994; Maeda *et al.,* 1997; Suzuki *et al.,* 1997). However, at midgastrulation (stage 12) pronounced differences between the ectodermal expression patterns of Dlx3 and Msx1 were noted. Dlx3 transcripts were observed at high levels in the most anterior region of the prospective neural plate, while Msx1 expression was undetectable (Figs. 1A–1F). Other differences were also noted, for example, Msx1 exhibited elevated expression in the lateral boundaries of the neural plate, while Dlx3 did not (Figs. 1A and 1B). Additionally, Dlx3 expression was not detected in the region surrounding the blastopore (Figs. 1E and 1F), while the Msx1 gene showed expression in ventral/lateral mesoderm throughout gastrulation.

The anterior expression patterns for Dlx3 and Msx1 correlate with spatial patterning of the anteriormost region of the dorsal ectoderm. The differential Msx1 expression extended posteriorly away from the prospective neural plate and was excluded from the prospective cement gland region. To further evaluate these spatial boundaries, double *in situ* hybridizations were performed. Analysis with the cement gland marker Xag1 in late stage 13/early stage 14 embryos showed that the expression domain of Xag1 at this stage falls entirely within the region of Dlx3 expression, but does not overlap with that of Msx1 expression (Figs. 1G and 1H). Double *in situ* hybridizations with BF-1 showed that this anterior neural fold marker is also expressed within the Dlx3 domain, near the posterior boundary (Fig. 1I), with a gap between BF-1 and Msx1 expression (Fig. 1J). These results demonstrate a differential pattern of Dlx3 and Msx1 gene expression in the anterior neural plate and indicate that the region that expresses Dlx3 but not Msx1 corresponds to the prospective cement gland.

The apparent overlap of Dlx3 and BF-1 gene expression is somewhat misleading, as shown in Fig. 2. Sagittal sections of whole-mount double *in situs* with these two probes revealed that the BF-1 gene is predominantly active in deep ectodermal cells at the anterior neural fold, whereas Dlx3 expression in this area is restricted to the superficial layer. Thus these two genes do not exhibit overlapping expression *in vivo.*

Differential Response of Dlx3 and Msx1 to BMP Signaling

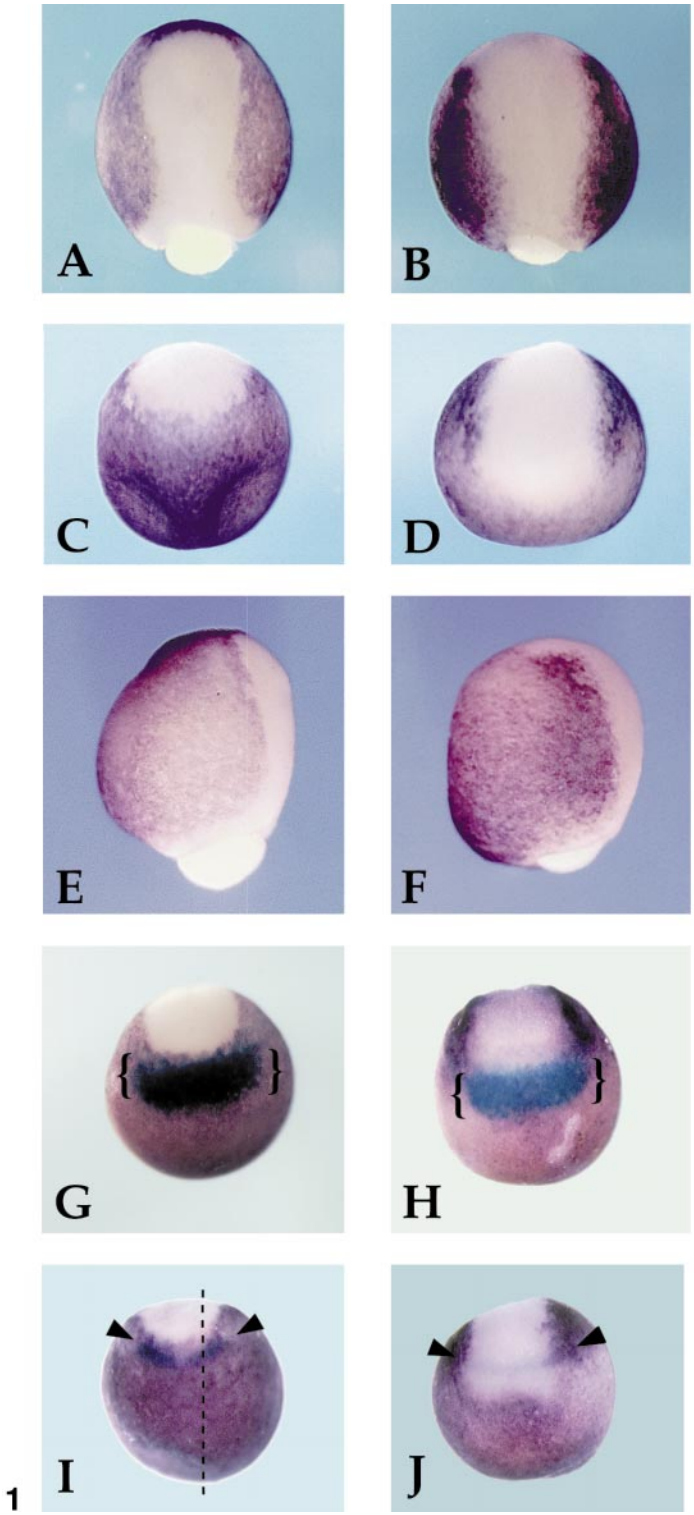
Expression of Dlx3 is localized to ventral ectoderm and thus might be expected to require BMP signaling for activation, like Msx1 and other genes transcribed in this region (Suzuki *et al.,* 1997). However, the striking difference in the posterior limit of Dlx3 versus Msx1 expression suggests that these two genes might respond differently to the embryonic BMP signaling gradient. This was investigated by a titration experiment in which the BMP signaling mechanism was gradually attenuated by injecting increasing doses of tBR RNA, followed by animal cap removal at stage 7–8, culture to stage 11, and RNA analysis for Dlx3 and Msx1 gene expression. As shown in Fig. 3, expression of Dlx3 is significantly less sensitive to reduction in BMP signaling than that of Msx1. For example, at a tBR dose of 500 pg, Msx1 gene expression was reduced approximately 90%, while relative Dlx3 expression remained at about 50% of the uninjected cap value. This suggests that a differential responsiveness to the BMP signal gradient along the anterior–posterior neural axis could account for the more posterior boundary of Dlx3 expression compared to that of Msx1. Moreover, since Dlx3 and Msx1 are transcriptional regulators, this could provide a molecular mechanism whereby the relative strength of the BMP signal is translated into differential gene expression in the developing anterior neural plate.

Dlx3 Is Not an Immediate-Early Response Gene

Another significant difference in the regulation of Dlx3 and Msx1 expression was revealed by an experiment in which animal caps were treated with cycloheximide to inhibit protein synthesis, prior to the onset of Dlx3 and Msx1 transcription. As shown in Fig. 4, this results in a drastic reduction in the Dlx3 RNA level at midgastrula, while Msx1 expression is actually increased. The latter result is consistent with earlier studies showing that Msx1 is an immediate-early response to BMP4 signaling (Suzuki *et al.,* 1997). Since the observed activation of Msx1 indicates that BMP signaling was intact in the cycloheximide-treated embryos, the most likely interpretation of this experiment is that transcriptional activation of Dlx3 requires protein synthesis following stimulation of the BMP signal transduction cascade. Note that the high Msx1 signal in whole embryos versus animal caps is due to the large fraction of expression of this gene in mesoderm, which is missing in the isolated animal cap.

Selective Repression of Neural Markers by Dlx3 and Msx1

Previous work has shown that inhibition of endogenous BMP signaling can affect cell fate decisions in the ectoderm. The inhibition of BMP signaling by morphogenic signals emanating from the organizer, such as chordin and noggin, can be mimicked experimentally by dissociating ectodermal cells (Godsave and Slack, 1989; Grunz and Tacke, 1989;



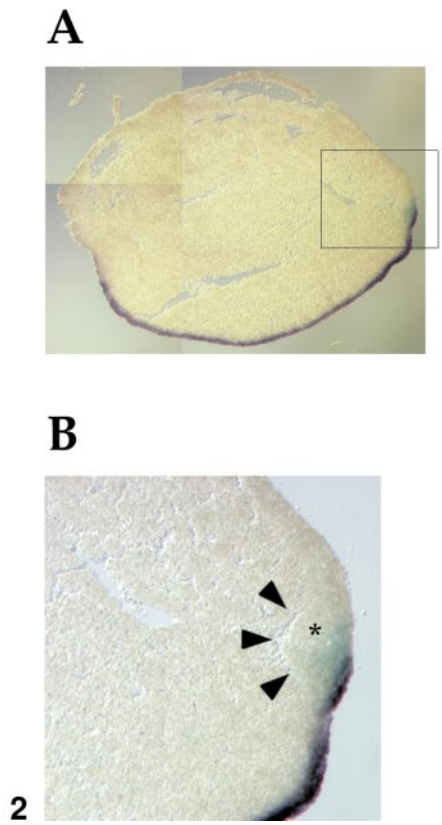


FIG. 1. Expression of Dlx3 and Msx1 at late gastrula. Whole, fixed albino embryos were hybridized *in situ* with probes for Dlx3 (A, C, E, G, I) or Msx1 (B, D, F, H, J). At stage 12 (A–F) the posterior boundary of Dlx3 expression was significantly closer to the blastopore than for Msx1. (A, B) Dorsal view (anterior toward top), (C, D) anterior view (dorsal toward top), (E, F) lateral view (dorsal to right). Double hybridizations *in situ* were performed with Xag1 (brackets, G, H) and BF-1 (arrowheads, I, J). In the extreme anterior neural plate region, both Xag1 and BF-1 transcripts were contained within the Dlx3 expression domain (G, I). In contrast, Xag1 and BF-1 expression was posterior to that of Msx1 (H, J). The dashed line in I corresponds to the plane of section shown in Fig. 2.

FIG. 2. Dlx3 and BF-1 are expressed in different strata of the anterior neurectoderm. An embryo which had undergone double *in situ* hybridization with Dlx3 (purple) and BF-1 (turquoise) was cryosectioned sagittally, as indicated by the dotted line in Fig. 1I. Arrows delineate the deep boundary of the anterior neural fold (asterisk). Dlx3 is expressed in the superficial cells, while BF-1 is expressed in the deep ectoderm. B is a magnification of the box shown in A.

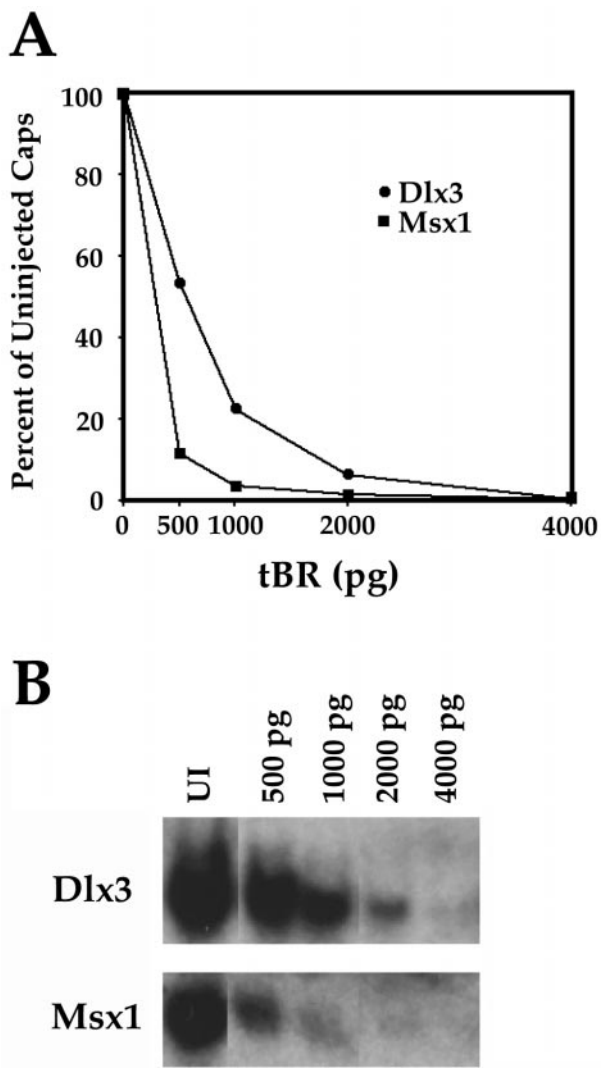


FIG. 3. Differential sensitivity of Dlx3 and Msx1 expression to inhibition of BMP signaling. Animal caps were isolated from embryos injected with 500, 1000, 2000, or 4000 pg of RNA encoding tBR, cultured until siblings reached stage 11, and analyzed for Dlx3 and Msx1 expression by Northern blotting. Dlx3 expression persists at significantly higher levels of tBR than does that of Msx1. (A) Graph summarizing densitometric analysis of multiple exposures of X-ray film, normalized to linear film response range. (B) Sample exposure.

Sato and Sargent, 1989) or by injection of either truncated BMP4 receptor (tBR; Graff *et al.,* 1994; Suzuki *et al.,* 1994) or antisense BMP4 RNA (Sasai *et al.,* 1995). All of these procedures result in the downregulation of epidermal gene expression and the activation of genes normally expressed in the anterior neurectoderm. More recently, Hemmati-Brivanlou and co-workers have proposed that a graded distribution of BMP signaling in the ectoderm is translated into regional specification, with low BMP activity corresponding to neural precursors, intermediate BMP activity resulting in cement gland and neural crest, and high BMP activity maintaining ventral identity (Wilson *et al.,* 1997). Based on our results, it seemed likely that differential levels of Dlx3 and Msx1 might have a role in specification of ectoderm receiving intermediate BMP levels. We tested this hypothesis by injecting RNA encoding tBR to generate neuralized ectodermal explants and co-injected RNA encoding Msx1 or Dlx3 to evaluate effects of these factors on neural gene expression. In these experiments, 2000 pg of tBR RNA and 120 pg of Msx1 or Dlx3 RNA were used. Lower doses of Msx1 and Dlx3 RNA lessened the extent of the inhibitory effects we observed, but did not alter the outcome qualitatively (data not shown). Higher doses resulted in dissociation, cell cycle arrest, and apoptosis and were not used in this study.

Animal caps were excised at midblastula (stage 7–8) and cultured until sibling embryos reached late neurula stage (stage 18). Marker gene expression was evaluated by Northern analysis. As shown in Fig. 5, injection with tBR alone resulted in the induction of expression of anterior neural markers and suppression of epidermal keratin, reflecting neuralization of ectoderm. Co-injection of Dlx3 with tBR inhibited activation of the anterior neural fold marker, BF-1 (Bourguignon *et al.,* 1998), the neural prepattern gene Zic3 (Nakata *et al.,* 1997), and the panneural marker Nrp1 (Richter *et al.,* 1990) but had no discernable effect on the anterior neural plate markers Otx2 (forebrain and cement gland; Blitz and Cho, 1995; Pannese *et al.,* 1995), CG7 (cement gland; Jamrich and Sato, 1989), or Xag1 (cement gland; Sive *et al.,* 1989). Co-injection of tBR with Msx1 also suppressed BF-1, but unlike Dlx3 this factor inhibited the activation of the anterior neural plate markers Otx2, CG7, and Xag1. The prepattern gene Zic3 was partially repressed, and the panneural marker Nrp1 appeared not to be significantly reduced in expression. These data suggest that the

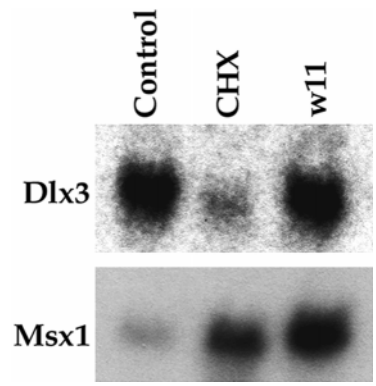


FIG. 4. Differential sensitivity of Dlx3 and Msx1 expression to inhibition of protein synthesis. Animal caps were isolated at stage 7 and cultured until stage 11 (midgastrula) in 0.3× MMR (Control) or 0.3× MMR + 5 μg/ml cycloheximide (CHX) and then analyzed by RNA blotting for Dlx3 and Msx1 expression. Dlx3 RNA accumulation was substantially blocked by this procedure, while Msx1 expression was superinduced. w11, whole stage 11.

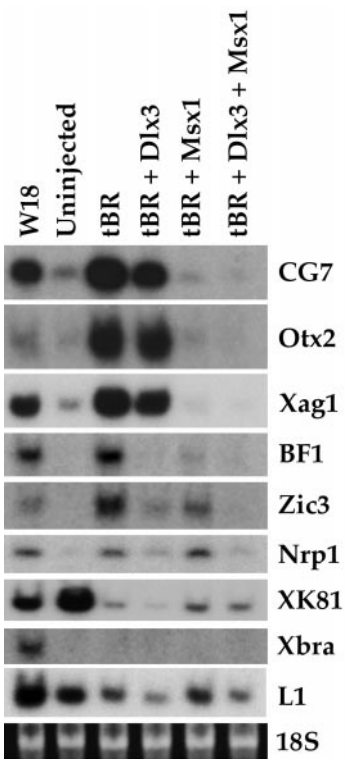


FIG. 5. Gene expression in animal caps injected with RNAs encoding tBR, Msx1, and Dlx3. Animal caps were removed from embryos injected with 2 ng tBR, 2 ng tBR+120 pg Dlx3, 2 ng tBR+120 pg Msx1 RNA, or 2 ng tBR+120 pg Dlx3+120 pg Msx1 or from uninjected embryos, cultured until sibling embryos reached late neurula (stage 18; W18), and processed for RNA extraction. Northern blot analysis revealed that Dlx3 was permissive for expression of the anterior neural marker Otx2 and the cement gland markers CG7 and Xag1, while Msx1 inhibited expression of these markers. Dlx3 suppressed expression of the panneural marker gene Nrp1, the "prepattern" gene Zic3, and the anterior neural fold gene BF1. Msx1 also inhibited Zic3 and BF1, but appeared to have less effect on Nrp1. The epidermal keratin gene XK81 was robustly expressed in uninjected caps and suppressed by tBR injection. Neither Dlx3 nor Msx1, nor a combination of both factors (last lane), increased XK81 expression significantly. Absence of signal with Xbra verified that the animal caps were not contaminated with mesoderm, and the accumulation of the ribosomal protein RNA L1 was used as a control for explant viability. At the bottom is a photograph of the ethidium bromide staining of the 18S region from a typical gel.

homeodomain genes Dlx3 and Msx1 function in a substantially complementary manner to selectively repress early neural markers. Interestingly, we did not observe significant recovery of epidermal-specific keratin gene expression in caps from tBR-injected embryos co-injected either with Dlx3 or with Msx1.

Dlx3 and Msx1 proteins share a high degree of similarity in their homeodomains and bind to essentially identical DNA sequences *in vitro* (Catron *et al.*, 1997; Feledy *et al.*, 1999). Furthermore, Msx1 is a transcriptional repressor while Dlx3 is an activator (Zhang *et al.*, 1997; Feledy *et al.*, 1999). Evidence from *in vitro* binding experiments and co-expression studies have suggested that Msx and Dlx homeoproteins act as mutual antagonists *in vivo* (Zhang *et al.*, 1997). However, as shown in Fig. 5, co-injection of Dlx3 and Msx1 into *Xenopus* embryos does not support this conclusion. On the contrary, the effects appear to be additive, resulting in complete inhibition of all neural marker genes tested. Interestingly, even under these conditions epidermal keratin gene expression was not significantly restored, suggesting that it is possible to prevent neural gene expression in BMP-inhibited ectoderm without reversion to an epidermal phenotype.

Dlx3 Functions Upstream of Zic3

Zic3 was identified in a differential screen for neural genes activated by chordin and suppressed by BMP4, and overexpression of this factor resulted in conversion of ectoderm to anterior neural tissue (Nakata *et al.*, 1997). The finding that Dlx3 was able to suppress the activation of Zic3 suggests that a Dlx3-mediated regulatory step might exist between the initial disruption of BMP signaling and the activation of this gene. To test this hypothesis tBR, Dlx3, and Zic3 RNAs were injected in combinations, followed by animal cap excision, culture, and RNA isolation for Northern blot analysis. As shown in Fig. 6A, Dlx3 blocked the activation of the panneural marker Nrp1 by tBR, similar to the result shown in Fig. 5. Addition of Zic3 RNA to the injection mixture restored Nrp1 expression to levels comparable to those of tBR-induced caps (Fig. 6B). Based on these results we conclude that the inductive effects of Zic3 function downstream of the antineurogenic step mediated by Dlx3.

Selective Inhibition of Anterior Neural Gene Expression by Dlx3 in the Intact Embryo

While the *Xenopus* animal cap assay has proven to be an invaluable tool in the study of early vertebrate development, it may not completely reflect events taking place in an intact embryo. To address this issue, a reduced dose (40–60 pg) of Dlx3 RNA was co-injected with β-galactosidase mRNA as a lineage tracer into one of the two dorsal animal blastomeres at the 8- to 16-cell stage. After culturing to stage 17, injected embryos and controls were fixed and stained with salmon-gal. Embryos exhibiting β-galactosidase activity on one side of the neural tube region underwent whole-mount *in situ* hybridization using probes for BF-1, Zic3, and Xag1. As shown in Fig. 7, targeted Dlx3 RNA injection resulted in disruption of the neural markers BF-1 and Zic3 but had no effect on Xag1. Overlying epidermis appeared unaffected, and no malformations of adjacent tissues such as notochord or somitic mesoderm were observed. Nor was there any apparent expansion of the Xag1 expression domain in Dlx3-injected embryos. Thus the effects of Dlx3 in the intact embryo are consistent with

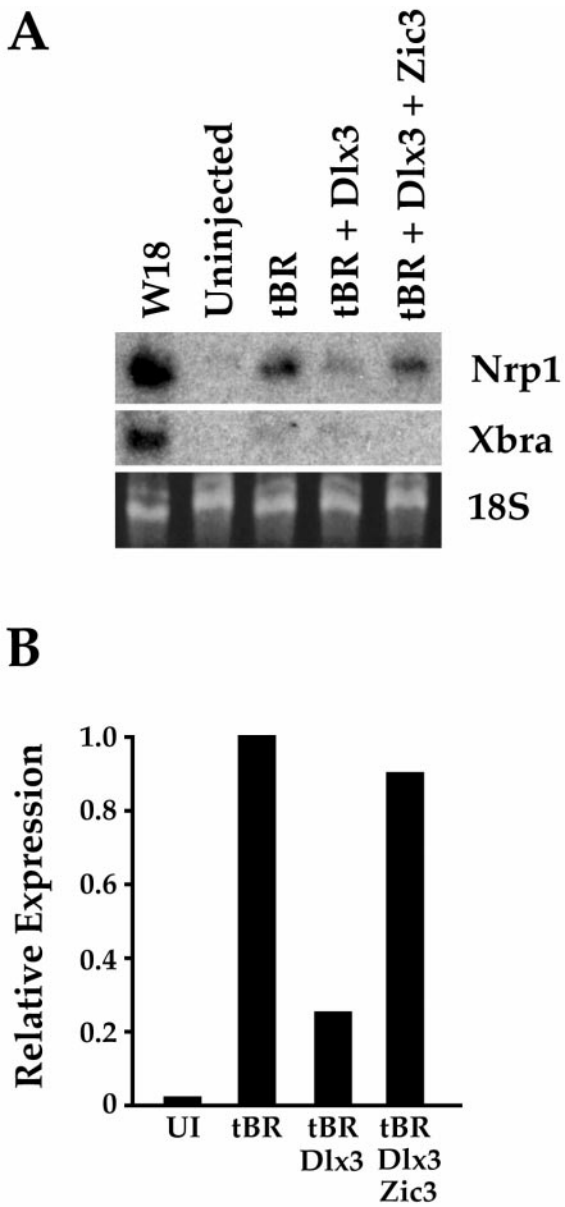


FIG. 6. Dlx3 functions upstream of Zic3. Animal caps were isolated from embryos injected with 2 ng tBR, 2 ng tBR+120 pg Dlx3, or 2 ng tBR+120 pg Dlx3+100 pg Zic3 RNA and from uninjected embryos, cultured until sibling embryos reached late neurula (W18; stage 18), and analyzed by Northern blot (A). Expression of the panneural marker Nrp1 was induced by tBR, inhibited by Dlx3, and restored by Zic3, indicating that Zic3 functions downstream of the negative regulatory step imposed by Dlx3. Ethidium bromide staining of the 18S rRNA region is shown as a gel loading control. Absence of hybridization to the Xbra probe indicated that the animal caps were not contaminated with mesoderm. (B) Histogram representing densitometry of Nrp1 hybridization signals.

the findings from experiments with ectodermal explants. Furthermore the inhibition of neural genes such as BF-1 and Zic3 does not result in respecification of anterior neural plate tissue as cement gland, suggesting that Dlx3 may play a permissive rather than instructive role in the formation of this nonneural tissue.

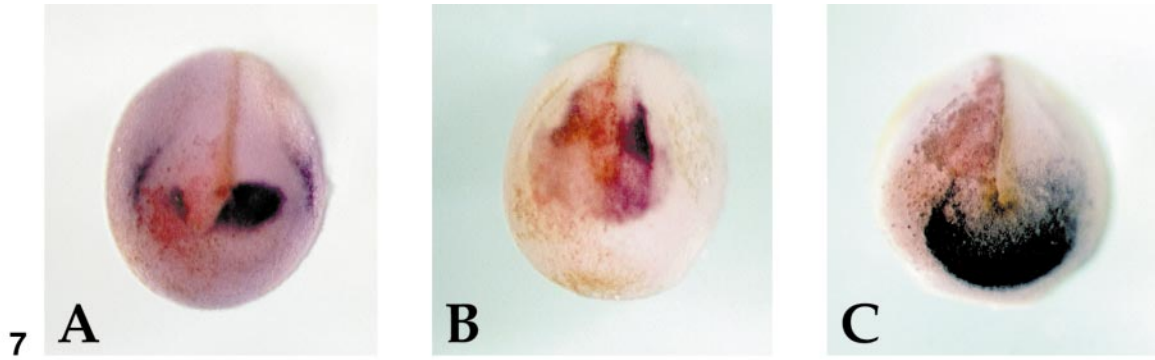
DISCUSSION

Translation of a BMP Signaling Gradient into Regionalized Gene Expression by Dlx3 and Msx1

The results presented in this paper show that Dlx3, like Msx1, can function as an antineural factor and that furthermore these homeodomain genes are differentially expressed in the anterior neural plate, probably in response to differential sensitivity to BMPs, and selectively inhibit the expression of distinct subsets of downstream target genes. This suggests a molecular mechanism whereby a morphogenic gradient of BMP activity, controlled by antagonizing factors secreted from the Spemann organizer, can pattern gene expression in the anterior region of the presumptive neural plate.

In *Xenopus* embryos, the predominant structure derived from the most anterior portion of the neural plate is the cement gland. This is a nonneural tissue, but like the rest of the anterior neural plate, is induced by inhibition of BMP signaling. As shown by Hemmati-Brivanlou and co-workers (Wilson *et al.,* 1997), optimal cement gland induction occurs at intermediate BMP levels; lower BMP concentrations, which would correspond to higher levels of neural inducers *in vivo,* result in the activation of more posterior, neural tissues and reduced induction of cement gland. As shown in Fig. 8, we can now explain this. As the organizer region moves anteriorly during gastrulation, secretion of anti-BMP factors should generate a moving gradient of BMP activity. At the end of gastrulation, this should result in a region, shown in tan color in Fig. 8, where Dlx3 (green) is expressed but Msx1 (magenta) is silenced. Based on the selective inhibition results shown in Fig. 5, this region should be permissive for gene expression associated with the cement gland and other nearby nonneural tissues and nonpermissive for neuralizing transcription factors such as Zic3 or BF-1. The intrusion of presumptive neural tissue into this region is limited to the deep ectodermal cells, as indicated by the light blue color in Fig. 8 and illustrated by the BF-1 expression shown in Fig. 2. Anterior to this intermediate zone is the epidermis, where both Msx1 and Dlx3 are expressed, and posterior to it, where neither homeodomain gene is active, is the prospective central nervous system. We note that in the vicinity of the blastopore at late gastrula there is also a region of nonoverlap in Msx1 and Dlx3 expression (Figs. 1A and 1B and indicated in Fig. 8). The significance of this is unknown, but it could be important in determining the fate of circumblastoporal ectoderm lying at the posterior end of the neural tube.

The observation (Fig. 7) that Dlx3 can interfere with expression of BF-1 and Zic3 in an intact embryo without a concomitant expansion of cement gland gene expression suggests that Dlx3 functions to create a permissive environment for cement gland rather than acting as a specifica-



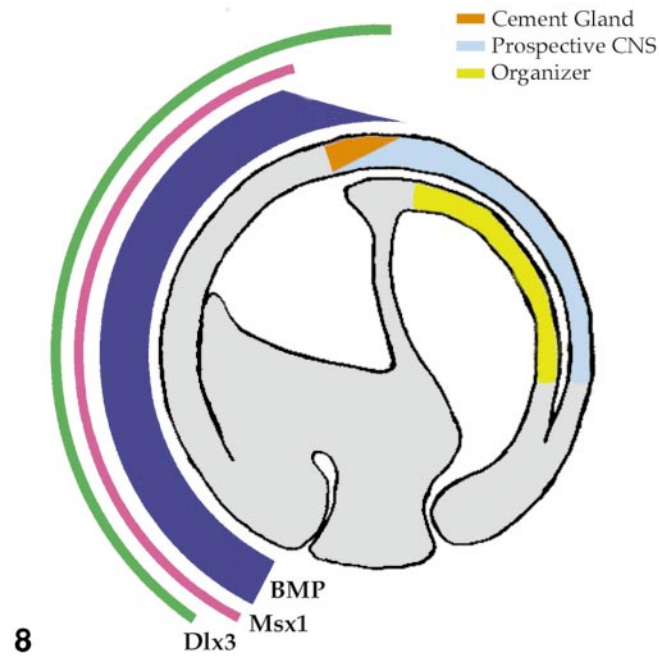


FIG. 7. Disruption of anterior neural gene expression *in vivo* by Dlx3. Embryos were injected in a single dorsal animal blastomere at the 8- to 16-cell stage with a mixture of RNA encoding Dlx3 (40–60 pg) and β-galactosidase (300 pg). At stage 17 embryos were fixed and stained briefly with salmon-gal to identify correctly targeted embryos, which were then hybridized *in situ* with probe for BF-1 (A), Zic3 (B), or Xag1 (C). The salmon-gal staining is pinkish-red, and the hybridization staining is purple. The co-injected side is to the left, and the uninjected right side serves as a control. Dlx3 injection repressed expression of BF-1 and Zic3, but had no discernable effect on Xag1.

FIG. 8. Model of ectodermal patterning by BMP-regulated expression of Dlx3 and Msx1. A sagittal section of a midgastrula embryo is depicted to illustrate the spatial relationships occurring along the dorsal–ventral neuraxis during gastrulation. Under the influence of the BMP signaling (purple), Msx1 (magenta) is expressed ventrally from a point near the blastopore lip to an anterior limit relatively distant from the leading edge of organizer tissue (yellow). Dlx3 (green) is also ventrally expressed but the domain extends from a point farther from the blastopore lip than Msx1 and extends more posteriorly, into a region with relatively greater attenuation of the BMP signal. In the extreme anterior neural plate, the ectoderm is divided into three domains: Msx1+/Dlx3+, which forms epidermis, Msx1−/Dlx+, giving rise predominantly to the cement gland (tan), and Msx1−/Dlx3−, which is initially specified as anterior prospective central nervous system tissue (light blue). Thus, differential activation of Dlx3 and Msx1 by intermediate levels of BMP activity results in patterning of the anteriormost dorsal ectoderm. Dorsal is to the right and anterior is up in this diagram.

tion factor per se. This is consistent with the inhibitory mode of action for both Dlx3 and Msx1 in modulating neural plate gene expression. Presumably the upregulation of tissue-specific gene expression in the cement gland involves other transcription factors that are not induced by Dlx3, such as Otx2 (Gammill and Sive, 1997).

Neural Inhibition by Dlx3 and Msx1 Does Not Restore Epidermis

It has become reasonably clear that in *Xenopus,* disruption of BMP signaling results in the conversion from epidermis to a neural fate (Hemmati-Brivanlou and Melton,

1997). This has led to the "default model" which suggests that ectoderm is programmed to become neural tissue and will differentiate into epidermis if this fate is prevented. Based on this model, it might be expected that inhibition of neural gene expression by introduction of BMP-dependent antineural factors, i.e., Dlx3 or Msx1, would result in the recovery of epidermis in ectoderm from embryos injected with tBR. However, our results do not support this.

As shown in Figs. 5 and 7, Dlx3 blocks expression of both regulatory and nonregulatory neural-specific genes in explants from tBR-injected embryos, but does not significantly restore the expression of epidermal keratin in the injected explants. In fact, as shown in Fig. 5, neither Dlx3 nor Msx1, nor a combination of both factors, increased keratin expression in explants from tBR-injected embryos significantly, compared to controls. With regard to Msx1, this is in conflict with some results of similar experiments reported earlier (Suzuki *et al.*, 1997). The reasons for this discrepancy are not clear. However, Suzuki *et al.* used an Msx1 construct including some 5′ and 3′ untranslated sequences, with multiple, in-frame potential translational initiation signals (Su *et al.*, 1991), whereas in our experiments a defined open reading frame with an optimized translational start site (Kozak, 1987) was used. Other technical differences, such as RNA injection dosages, or the use of RT-PCR versus Northern blot analysis could also be contributing factors. It is not likely, however, that the low keratin expression in our experiments is due to any generalized inhibition of biosynthetic activity in the explants, since the accumulation of the ribosomal protein RNA control (L1, Fig. 5) was not markedly inhibited in any of the explant samples. We conclude that Msx1 has little capacity to restore epidermal gene expression to control levels in a tBR-injected animal cap and that Dlx3 has none. We also conclude that most if not all neural gene expression can be inhibited in ectoderm from tBR-injected embryos without leading to restoration of epidermis.

This suggests that something else in addition to Dlx3 and Msx1 must be required in order for ectoderm to differentiate as epidermis. Such a factor or factors would presumably be dependent on BMP signaling and might be expected to reside specifically in ventral tissues similar to Dlx3 and Msx1. Some candidate genes have been described, such as XVent family members (Ault *et al.*, 1997; Onichtchouk *et al.*, 1998) and Gata1b (Xu *et al.*, 1997), that may fulfill such a role, but it is possible that additional uncharacterized factors are involved. Another unanswered question is how Dlx3, which is a strong transcriptional activator, can act as a repressor of neural gene expression. One possibility is that Dlx3 interacts with modifying factors to yield a heteromeric complex that now functions as a repressor. Alternatively, Dlx3 might activate the expression of negative regulatory factors.

In conclusion, differential regulation of Dlx3 and Msx1 by a BMP morphogenic gradient provides a potential molecular mechanism for translating organizer-dependent spatial information into localized gene expression in the developing anterior neural plate. It would also appear that the specification of epidermis is likely to require positive regulation by BMP-dependent transcription factors, in addition to the inhibition of the neural "default" state by Dlx3 and Msx1.

ACKNOWLEDGMENTS

We thank Francesco Ramirez for *Xenopus* Msx1, Katsuhiko Mikoshiba for Zic3, Nancy Papalopulu for BF-1, and Naeto Ueno for tBR plasmids; Michael Tsang for advice on *in situ* hybridization procedures; Sally Moody for helpful discussions; and Ankur Gupta and Timur Yusufzai for assistance in preparing constructs for RNA synthesis and other contributions.

REFERENCES

Ault, K. T., Xu, R., Kung, H., and Jamrich, M. (1997). The homeobox gene PV.1 mediates specification in the prospective neural ectoderm in *Xenopus* embryos. *Dev. Biol.* **192,** 162–171.

Bailey, J. M., and Davidson, N. (1976). Methyl-mercury as a reversible denaturing agent for agarose gel electrophoresis. *Anal. Biochem.* **70,** 75–85.

Blitz, I. L., and Cho, K. W. (1995). Anterior neurectoderm is progressively induced during gastrulation: The role of the *Xenopus* homeobox gene orthodenticle. *Development* **121,** 993–1004.

Bourguignon, C., Li, J., and Papalopulu, N. (1998). *BF-1*, a winged helix transcription factor with dual activity, has a role in positioning neurogenesis in *Xenopus* competent ectoderm. *Development* **125,** 4889–4900.

Brewster, R., Lee, J., and Ruiz i Altaba, A. (1998). Gli/Zic factors pattern the neural plate by defining domains of cell differentiation. *Nature* **393,** 579–583.

Catron, K. M, Iler, N., and Abate, C. (1993). Nucleotides flanking a conserved TAAT core dictate the DNA binding specificity of three murine homeodomain proteins. *Mol. Cell. Biol.* **13,** 2354–2365.

Cohen, S. M., Bronner, G., Kutter, F., Jurgens, G., and Jackle, H. (1989). Distal-less encodes a homeodomain protein required for limb development in *Drosophila*. *Nature* **338,** 432–434.

Davidson, D. (1995). The function and evolution of Msx genes: Pointers and paradoxes. *Trends Genet.* **11,** 405–411.

Dirksen, M.-L., Morasso, M. I., Sargent, T. D., and Jamrich, M. (1994). Differential expression of a Distal-less homeobox gene Xdll-2 in ectodermal cell lineages. *Mech. Dev.* **46,** 63–70.

Fainsod, A., Deissler, K., Yelin, R., Marom, K., Epstein, M., Pillemer, G., Steinbeisser, H., and Blum, M. (1997). The dorsalizing and neural inducing gene follistatin is an antagonist of BMP-4. *Mech. Dev.* **63,** 39–50.

Feledy, J. A., Morasso, M. I., Jang, S.-I., and Sargent, T. D. (1999). Transcriptional activation by the homeodomain protein Distal-less 3. *Nucleic Acids Res.* **27,** 764–770.

Gammill, L., and Sive, H. (1997). Identification of Otx2 target genes in restriction in ectodermal competence during *Xenopus* cement gland formation. *Development* **124,** 471–481.

Godsave, S. F., and Slack, J. M. W. (1989). Clonal analysis of mesoderm induction in *Xenopus laevis*. *Dev. Biol.* **134,** 486–480.

Graff, J. M. (1997). Embryonic patterning: To BMP or not to BMP, that is the question. *Cell* **89,** 171–174.

Graff, J. M., Thies, R. S., Song, J. J., Celeste, A. J., and Melton, D. A. (1994). Studies with a *Xenopus* BMP receptor suggest that ventral

mesoderm-inducing signals override dorsal signals in vivo. *Cell* **79,** 169–179.

Grunz, H., and Tacke, L. (1989). Neural differentiation of *Xenopus laevis* ectoderm takes place after dis-aggregation and delayed reaggregation without inducer. *Cell Differ. Dev.* **28,** 211–218.

Harland, R. M. (1991). *In situ* hybridization: An improved whole-mount method for *Xenopus* embryos. *Methods Cell Biol.* **36,** 685–695.

Hemmati-Brivanlou, A., and Melton, D. (1997). Vertebrate embryonic cells will become nerve cells unless told otherwise. *Cell* **88,** 13–17.

Hogan, B. L. M. (1996). Bone morphogenetic proteins: Multifunctional regulators of vertebrate development. *Genes Dev.* **10,** 1580–1594.

Jonas, E., Sargent, T. D., and Dawid, I. B. (1985). Epidermal keratin gene expressed in embryos of *Xenopus laevis*. *Proc. Natl. Acad. Sci. USA* **82,** 5413–5417.

Kozak, M. (1987). An analysis of noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids. Res.* **15,** 8125–8148.

Lee, J. E., Hollenberg, S. M., Snider, L., Turner, D. L., Lipnick, N., and Weintraub, H. (1995). Conversion of *Xenopus* ectoderm into neurons by NeuroD, a basic helix-loop-helix protein. *Science* **268,** 836–844.

Ma, Q., Kintner, C., and Anderson, D. (1996). Identification of neurogenin, a vertebrate neuronal determination gene. *Cell* **87,** 43–52.

Maeda, R., Kobayashi, A., Ryo, S., Lin, J. J., Kung, H., and Maeno, M. (1997). Xmsx-1 modifies mesodermal tissue pattern along dorsoventral axis in *Xenopus laevis* embryo. *Development* **124,** 2553–2560.

Mizuseki, K., Kishi, M., Matsui, M., Nakanishi, S., and Sasai, Y. (1998). *Xenopus* Zic-related-1 and Sox-2, two factors induced by chordin, have distinct activities in the initiation of neural induction. *Development* **125,** 579–587.

Morasso, M. I., Markova, N. G., and Sargent, T. D. (1996). Regulation of epidermal differentiation by a Distal-less homeodomain gene. *J. Cell. Biol.* **135,** 1879–1887.

Nakata, K., Nagai, T., Aruga, J., and Mikoshiba, K. (1997). *Xenopus* Zic3, a primary regulator both in neural and neural crest development. *Proc. Natl. Acad. Sci. USA* **94,** 11980–11985.

Nieuwkoop, P. D., and Faber, J. (1967). "Normal Table of *Xenopus laevis* (Daudin)." North-Holland, Amsterdam.

Onichtchouk, D., Glinka, A., and Niehrs, C. (1998). Requirement for Xvent-1 and Xvent-2 gene function in dorsoventral patterning of *Xenopus* mesoderm. *Development* **125,** 1447–1456.

Pannese, M., Polo, C., Andreazzoli, M., Vignali, R., Kablar, B., Barsacchi, G., and Boncinelli, E. (1995). The *Xenopus* homologue of Otx2 is a maternal homeobox gene that demarcates and specifies anterior body regions. *Development* **121,** 707–720.

Piccolo, S., Sasai, Y., Lu, B., and De Robertis, E. M. (1996). Dorsoventral patterning in *Xenopus:* Inhibition of ventral signals by direct binding of chordin to Bmp-4. *Cell* **86,** 589–598.

Richter, K., Good, P. J., and Dawid, I. B. (1990). A developmentally regulated, nervous system-specific gene in *Xenopus* encodes a putative RNA-binding protein. *New Biol.* **2,** 556–565.

Sargent, T. D., Jamrich, M., and Dawid, I. B. (1986). Cell interactions and the control of gene activity during early development of *Xenopus laevis*. *Dev. Biol.* **114,** 238–246.

Sasai, Y. (1998). Identifying the missing links: Genes that connect neural induction and primary neurogenesis in vertebrate embryos. *Neuron* **21,** 455–458.

Sasai, Y., Lu, B., Steinbeisser, H., and De Robertis, E. M. (1995). Regulation of neural induction by the Chd and Bmp-4 antagonistic patterning signals in *Xenopus*. *Nature* **376,** 333–336.

Sato, S. M., and Sargent, T. D. (1989). Development of neural inducing capacity in dissociated *Xenopus* embryos. *Dev. Biol.* **134,** 263–266.

Sive, H., Hattori, K., and Weintraub, H. (1989). Progressive determination during formation of the anteroposterior axis in *Xenopus laevis*. *Cell* **58,** 171–180.

Smith, J. C., Price B. M., Green, J. B., Weigel, D., and Herrmann, B. G. (1991). Expression of a *Xenopus* homolog of Brachyury (T) is an immediate-early response to mesoderm induction. *Cell* **67,** 79–87.

Su, M. W., Suzuki, H. R., Solursh, M., and Ramirez, F. (1991). Progressively restricted expression of a new homeobox-containing gene during *Xenopus laevis* embryogenesis. *Development* **111,** 1179–1187.

Sudarwati, S., and Nieuwkoop, P. D. (1971). Mesoderm formation in the anuran *Xenopus laevis* (Daudin). *Wilhelm Roux Arch.* **166,** 189–204.

Suzuki, A., Theis, R. S., Yamaji, N., Song, J. J., Wozney, J., Murakami, K., and Ueno, N. (1994). A truncated BMP receptor affects dorsal–ventral patterning in the early *Xenopus* embryo. *Proc. Natl. Acad. Sci. USA* **91,** 10255–10259.

Suzuki, A., Ueno, N., and Hemmati-Brivanlou, A. (1997). *Xenopus* msx1 mediates epidermal induction and neural inhibition by BMP4. *Development* **124,** 3037–3044.

Turner, D. L., and Weintraub, H. (1994). Expression of achaete-scute homolog 3 in *Xenopus* embryos converts ectodermal cells to a neural fate. *Genes Dev.* **8,** 1434–1447.

Wilson, P. A., Lagna, G., Suzuki, A., and Hemmati-Brivanlou, A. (1997). Concentration-dependent patterning of the *Xenopus* ectoderm by BMP4 and its signal transducer Smad1. *Development* **124,** 3177–3184.

Xu, R., Kim, J., Taira, M., Lin, J., Zhang, C., Sredni, D., Evans, T., and Kung, H. (1997). Differential regulation of neurogenesis by the two *Xenopus* GATA-1 genes. *Mol. Cell. Biol.* **17,** 436–443.

Zhang, H., Hu, G., Wang, H., Sciavolino, P., Iler, N., Shen, M. M., and Abate-Shen, C. (1997). Heterodimerization of Msx and Dlx homeoproteins results in functional antagonism. *Mol. Cell. Biol.* **17,** 2920–2932.

Zimmerman, L. B., DeJesus-Escobar, J. M., and Harland, R. M. (1996). The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell* **86,** 599–606.

Zimmerman, K., Shih, J., Bars, J., Collazo, A., and Anderson, D. J. (1993). XASH-3, a novel *Xenopus* achaete-scute homolog, provides an early marker of planar neural induction and position along the mediolateral axis of the neural plate. *Development* **119,** 221–232.

Received for publication December 21, 1998
Revised May 25, 1999
Accepted June 9, 1999